

REPORT DOCUMENTATION PAGE

Form Approved
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1. AGENCY USE ONLY <i>(Leave blank)</i>	2. REPORT DATE 18 October 2002	3. REPORT TYPE AND DATES COVERED	
4. TITLE AND SUBTITLE Determination of the virulence of the pigmentation-deficient and pigmentation-/plasminogen activator-deficient strains of <i>Yersinia pestis</i> in non-human primate and mouse models of pneumonic plague		5. FUNDING NUMBERS	
6. AUTHOR(S) S. Welkos, M.L.M. Pitt, M. Martinez, A. Friedlander, P. Vogel, R. Tammaro			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research Institute of Infectious Diseases 1425 Porter St Fort Detrick MD 21702-5011		8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)		10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES			
12a. DISTRIBUTION / AVAILABILITY STATEMENT APPROVED FOR PUBLIC RELEASE DISTRIBUTION UNLIMITED		12b. DISTRIBUTION CODE	
13. ABSTRACT <i>(Maximum 200 words)</i> The current human plague vaccine, a killed <i>Yersinia pestis</i> whole-cell preparation, does not protect against aerosol challenge and is reactogenic and antigenically undefined. Live attenuated <i>Y. pestis</i> , such as pigmentation-deficient (Pgm-) strains, have been used frequently as vaccines and are efficacious. They are used widely in plague research and assumed to be safe. However, they can cause serious adverse reactions, and their aerosol infectivity is not known. We tested the virulence of a defined Pgm- variant of the C092 strain of <i>Y. pestis</i> in mouse and non-human primate models of pneumonic plague. The ten-fold lower median lethal dose by the aerosol compared to the subcutaneous (s.c.) routes of the Pgm -strain in mice suggested that the Pgm -strain might be less attenuated by the former than by the latter route. After exposure of 16 African green monkeys to inhaled doses ranging from 1.1×10^4 to 8.1×10^7 cfu, eight died and eight survived. The terminal cultures collected from five of the non-survivors were all positive for <i>Y. pestis</i> . Two of the remaining three non-survivors were culture-negative but had pathologic and immunologic evidence of infection with <i>Y. pestis</i> , specimens could not be obtained nor the cause of death determined for the third one. The deaths were not dose-related, and there were some differences in the pathology associated with infection by the Pgm- strain compared to the wild-type (wt) strain. However, the Pgm- derivative was clearly virulent for monkeys by the aerosol route. A mutant of the Pgm- strain, which has a deletion in the plasminogen activator (Pla) virulence locus (pla), appeared to be more attenuated than was either the Pgm- single mutant (in NHPs and mice) or the Pla- single mutant strain (in mice) and has potential as a live vaccine.			
14. SUBJECT TERMS Aerosol; Plague; <i>Yersinia pestis</i> ; Primates; Pigmentation locus; <i>Pla</i> ; Live vaccine		15. NUMBER OF PAGES 9	
		16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT UNCLASSIFIED	18. SECURITY CLASSIFICATION OF THIS PAGE UNCLASSIFIED	19. SECURITY CLASSIFICATION OF ABSTRACT UNCLASSIFIED	20. LIMITATION OF ABSTRACT UNLIMITED

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20021122066



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Vaccine 20 (2002) 2206–2214

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Determination of the virulence of the pigmentation-deficient and pigmentation-/plasminogen activator-deficient strains of *Yersinia pestis* in non-human primate and mouse models of pneumonic plague[☆]

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Received 28 October 2001; received in revised form 14 January 2002; accepted 8 February 2002

Abstract

The current human plague vaccine, a killed *Yersinia pestis* whole-cell preparation, does not protect against aerosol challenge and is reactogenic and antigenically undefined. Live attenuated *Y. pestis*, such as pigmentation-deficient (*Pgm*[−]) strains, have been used frequently as vaccines and are efficacious. They are used widely in plague research and assumed to be safe. However, they can cause serious adverse reactions, and their aerosol infectivity is not known. We tested the virulence of a defined *Pgm*[−] variant of the C092 strain of *Y. pestis* in mouse and non-human primate models of pneumonic plague. The ten-fold lower median lethal dose by the aerosol compared to the subcutaneous (s.c.) routes of the *Pgm*[−] strain in mice suggested that the *Pgm*[−] strain might be less attenuated by the former than by the latter route. After exposure of 16 African green monkeys to inhaled doses ranging from 1.1×10^4 to 8.1×10^7 cfu, eight died and eight survived. The terminal cultures collected from five of the non-survivors were all positive for *Y. pestis*. Two of the remaining three non-survivors were culture-negative but had pathologic and immunologic evidence of infection with *Y. pestis*, specimens could not be obtained nor the cause of death determined for the third one. The deaths were not dose-related, and there were some differences in the pathology associated with infection by the *Pgm*[−] strain compared to the wild-type (wt) strain. However, the *Pgm*[−] derivative was clearly virulent for monkeys by the aerosol route. A mutant of the *Pgm*[−] strain, which has a deletion in the plasminogen activator (*Pla*) virulence locus (*pla*), appeared to be more attenuated than was either the *Pgm*[−] single mutant (in NHPs and mice) or the *Pla*[−] single mutant strain (in mice) and has potential as a live vaccine. Published by Elsevier Science Ltd.

Keywords: Aerosol; Plague; *Yersinia pestis*; Primates; Pigmentation locus; *Pla*; Live vaccine

1. Introduction

The human plague vaccine (Plague Vaccine USP), a killed *Yersinia pestis* whole-cell preparation, partially protected mice against subcutaneous (s.c.) infection, but did not protect mice or monkeys against aerosol challenge ([1–5]; Pitt, et al., unpublished data). Also, the vaccine is reactogenic, antigenically undefined, and requires several booster doses to achieve elevated antibody (Ab) levels. Component vaccine candidates containing purified fraction 1 capsule (F1)

antigen and the plasmid pLcr-encoded V (“virulence”) antigen (V) of *Y. pestis* appear to be safe and protective against bubonic and pneumonic plague in mice. However, F1-based immunity does not protect animals against strains which are F1-negative but still virulent [2,4,6–8], and the response to V might not protect against challenge with strains producing serologic variants of V [9]. A live vaccine, in which a spectrum of potentially protective antigens would be present, might provide broader and more long-term protection.

Live attenuated *Y. pestis* strains have been used effectively as vaccines. Most of these attenuated strains were pigmentation-deficient (*Pgm*[−]), usually due to the spontaneous deletion of a 102 kb chromosomal fragment encoding iron binding and transport functions [10]. Live *Pgm*[−] strains were often more immunogenic than killed vaccines in animals, but they sometimes caused local and systemic

[☆] The views, opinions, and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

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reactions [11–13]. Reactogenicity varied with the host animal and the route of inoculation. For instance, such strains are virulent for mice by the intravenous (i.v.) route but attenuated by peripheral routes of infection [14], and several species of non-human primates are significantly more sensitive to *Pgm*[−] *Y. pestis* than are guinea pigs [13]. The aerosol infectivity of such strains is not known. Cultures of *Y. pestis* strains presumably isolated from patients during a recent outbreak of pneumonic and bubonic infections in India and available for study by collaborative centers appeared to be *Pgm*[−] ([15–17]; Chu, et al., unpublished). Nevertheless, studies done in the former Soviet Union showed that vaccinating humans by aerosol with a *Pgm*[−] strain is simple, safe, and effective [18–20].

Plasminogen activator (Pla) is encoded by the *pla* gene on plasmid pPst in *Y. pestis* [21]. Strains cured of pPst or having a deletion in *pla* are virulent by the i.v. route but are significantly attenuated after s.c. inoculation [21,22]. Plasmid pPst-cured and *Pla*[−] derivatives of C092 replicated at the site of infection in mice infected at a s.c. site, but showed only transient replication at peripheral sites [22]. These mutant strains might stimulate a vigorous immune response, and as Pla was not protective in mice (Sample et al., unpublished data), its absence should not decrease the efficacy of a live vaccine.

In this study, we examined the virulence by the aerosol route of the C092 *Pgm*[−] strain in a non-human primate (*Chlorocebus aethiops*) model of human pneumonic plague [14]. The lethality of a *Pla*[−] derivative of this strain for monkeys was then determined. Such defined, multiply-attenuated strains may be potentially safe and efficacious vaccine candidates.

2. Materials and methods

2.1. Animals

Twenty-one 5.3–7.0 kg adult male African green monkeys (*Chlorocebus aethiops*), and female 7–9-week-old outbred Swiss Webster mice obtained from Harlan Sprague Dawley (Indianapolis, IN), were used in these studies. Ten mice were used in each dose group for all lethal dose determinations, except five per dose for the s.c. challenges with the C092/*Pgm*[−] *Pla*[−] double mutant strain. All animals used in this research were cared for and used humanely according to the following policies: the US Public Health Service Policy on humane care and use of animals (1996); The Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animals Resources, National Research Council (ISBN 0-309-05377-3, revised 1996); and the US Government Principles for Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training (1985). All USAMRIID animal facilities and program are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All animal use was approved by the Institutional Animal Care and Use Committee

Table 1
Characteristics of strains

Strain (C092)	Relevant properties ^a
wt	Virulent wt strain, from human case of pneumonic plague
<i>Pgm</i> [−] ^b	C092 with a spontaneous deletion of the 102 kb chromosomal <i>pgm</i> locus.
pPst [−]	C092 cured of plasmid pPst which harbors <i>pla</i> , the gene encoding the 34–37 kDa Pla protease
<i>Pla</i> [−]	C092 with a frameshift mutation/deletion in <i>pla</i> . Mutated gene encodes a truncated, enzymatically inactive polypeptide of 26 kDa.
<i>Pgm</i> [−] , <i>Pla</i> [−]	C092 <i>Pla</i> [−] with deleted <i>pgm</i> locus.

^a The strains are described in more detail in Welkos et al. [22].

^b C092 *Pgm*[−] was isolated as a spontaneous pigmentation-deficient mutant in a culture of the wt strain on CR agar plates. It has a deletion of the entire 102 kb *Pgm* locus as determined by PCR analysis using primers to three genes that span the *Pgm* locus, *psn*, *irp2*, and *hms* (Worsham, unpublished).

and conducted in accordance with federal Animal Welfare Act regulations.

2.2. Bacterial strains and preparation of inocula

Bacterial strains used in this study are described in Table 1 and were stored in single-use aliquots at −70 °C in 66% glycerol. For challenge studies, frozen stocks were streaked onto tryptose blood agar base slants and incubated at 28 °C for 2 days. For challenges by the s.c. route, the bacterial growth was harvested from the slants in 5 ml heart infusion broth (HIB), adjusted to an OD₆₂₀ of 1.0 (approximately 10⁹ cfu/ml), diluted to the required concentration(s), and injected s.c. in 0.2 ml volumes [4]. The inocula for aerosol exposures were prepared as described elsewhere [4,8] from 100 ml HIB flask cultures incubated for 24 h in a 30 °C shaker at 100 rpm. The cultures were centrifuged and washed in HIB, adjusted to an OD₆₂₀ of 10.0 (approximately 1 × 10¹⁰ cfu/ml), and diluted to the desired aerosol dose. The final concentration of all inocula was determined by preparing 10-fold dilutions, spreading aliquots on sheep blood agar plates (SBAP), incubating the plates for 2 days at room temperature, and counting the colonies.

2.3. Aerosol and subcutaneous exposures

Monkeys were anesthetized and respiratory minute volumes measured immediately before challenge as described elsewhere [8]. The monkeys were individually exposed in a head-only chamber to an aerosol of *Y. pestis* generated by a three-jet collision nebulizer [8,23,24]. The aerosol was sampled continuously by an all-glass impinger (AGI, Ace Glass Inc., Vineland, NJ) containing HIB. For each animal, the aerosol concentration of bacteria and inhaled dose were calculated by plating out dilutions of a sample from the AGI onto SBAP. Mice were exposed s.c. and by aerosol to *Y. pestis* as described previously [4,25].

2.4. Necropsy, pathology, and tissue processing

Complete necropsies were performed on all monkeys that died after exposure to *Y. pestis*. Cardiac blood was aseptically collected for blood culture and for serum to measure antibody titers. Tissue samples were either aseptically collected and placed in sterile containers for culture, or were fixed in 10% neutral buffered formalin for histopathological processing as described previously [8]. Antigenic reactivity of selected tissue sections to a monospecific polyclonal rabbit anti-F1 serum was evaluated by using an avidin-biotin-peroxidase immunohistochemistry procedure [8]. The specimens cultured included blood, lung, liver, spleen, and any tissues that exhibited gross lesions at necropsy. The lung, spleen, and liver specimens for culture were first ground manually in a tissue grinder in approximately 1 ml of saline. All the specimens were plated on SBAP and Congo Red agar (CR) plates. The plates were incubated for 2–3 days at 25–30°C and the colonies examined morphologically and microscopically to verify the identification of *Y. pestis*.

2.5. Antibody titers

Serum antibody titers were determined by direct IgG enzyme-linked immunosorbent assay (ELISA), as described previously for the YopH antigen [26] and the F1 and V antigens [27], but using goat anti-monkey IgG, peroxidase-labelled secondary Ab. The endpoint titers were determined by the highest serum dilution with an OD₄₀₅ of ≥ 0.20 after subtraction of the OD₄₀₅ of the blank wells with no antigen.

3. Results

3.1. The virulence of the C092 Pgm[−] strain of *Y. pestis* in mice

In Swiss Webster mice, the C092 Pgm[−] strain was lethal at high s.c. doses only, with a LD₅₀ of 10^7 cfu (Table 2). A dose of 10^6 cfu (0.1 LD₅₀) of the C092 Pgm[−] strain given subcutaneously was generally nonlethal but still permitted limited replication, as detected by spleen culture. This dose also protected mice against s.c. challenge with the fully virulent strain, however, vaccine-related deaths occurred occasionally, and induration was sometimes observed at the site of inoculation in surviving vaccinees (data not shown).

The virulence of Pgm[−] strains by the aerosol route in animals has not been well characterized. Despite the relatively high s.c. LD₅₀ exhibited by the C092 Pgm[−] strain in mice (Table 2), its LD₅₀ aerosol dose was about a magnitude less (10^6 cfu, Table 2). The potentially reduced attenuation of virulence of Pgm[−] strains when inhaled as compared to being injected peripherally warranted further study.

Table 2
Virulence for mice of the wt, Pgm[−] and Pla[−] strains of C092

Strain (C092)	LD ₅₀ (number of cfu) by route ^a		
	s.c.	i.p.	Aerosol
wt	1.9	14.0	2.3×10^4
Pgm [−]	$\sim 10^7$	nd ^b	$\sim 10^6$
pPst [−]	1.4×10^6	7.6	10^5
Pla [−]	3.4×10^4	nd	nd
Pgm [−] , Pla [−]	$>10^8$ ^c	nd	nd

^a The s.c. and i.p. LD₅₀ values for strains C092 wt, C092 pPst[−] and C092 Pla[−] were reported previously by Welkos et al. [22]. The s.c. and aerosol challenges were all done using 10 mice per dose group, except five mice per dose was used for s.c. challenges with C092 Pgm[−] Pla[−].

^b nd:Not done.

^c All mice challenged with the highest dose tested, 1×10^8 cfu, survived.

3.2. The virulence of the C092 Pgm[−] strain of *Y. pestis* in monkeys exposed by aerosol

The virulence of the C092 Pgm[−] strain in 16 monkeys exposed by aerosol was characterized and is summarized in Tables 3 and 4. Eight of the monkeys exposed to doses ranging from 1.1×10^4 to 8.1×10^7 cfu died, survivors and non-survivors did not differ in weight (Tables 3 and 4). The deaths were not dose-related, however, the cause of death in seven of the eight non-survivors for which specimens were available for evaluation was attributed to the *Y. pestis* infection based on bacteriologic and/or pathologic findings (Table 3). Bacteriologic and/or antigenic evidence of infection by *Y. pestis* was present in the majority of the animals lethally infected with C092 Pgm[−] (88%) and, as reported previously [8], in all of the animals infected with the wild-type (wt) strain (Tables 3 and 5). Five of seven monkeys (71%) for which specimens could be obtained were positive for culture of *Y. pestis* Pgm[−], and seven of eight (88%) of lethally exposed animals were positive immunohistochemically for the presence of the F1 antigen. One of the two culture-negative, F1 antigen-positive animals (#3) developed necrotizing colitis with peritonitis and had to be killed on day 25. Although we were unable to demonstrate F1 antigen in the tissue section containing the colonic lesion, several other tissue sections stained strongly for F1 antigen and/or contained lesions compatible with those of other *Y. pestis* Pgm[−] strain-infected animals.

The pathology of lethal infection associated with the Pgm[−] strain resembled that caused by the virulent wt parent, however, we also observed notable differences (Table 5 and Figs. 1–4). Of the eight animals (12.5%) exposed to a lethal dose of the Pgm[−] strain, only one (Fig. 2a) exhibited a bronchopneumonia resembling that typically observed in animals infected with wt C092 (Fig. 1c), the sample shown in Fig. 1c is a photomicrograph taken recently of the lung sample from a monkey exposed in a previous study [8] to a lethal dose of wt C092. Instead, vascular leakage was a common finding in the lungs of the

Table 3
Virulence of C092 Pgm[−] for monkeys by the aerosol route and course of infection in non-survivors

Monkey ^a	Weight (kg)	Inhaled dose (cfu)	Culture results ^b				Comments	
			TTD, d ^c	Blood	Lung	Spleen		
1	5.8	0.9 × 10 ⁴	11	+	+	+	+	Pulmonary perivascular edema; myocardial necrosis; gastrointestinal, cerebral hemorrhage
2	5.8	1.1 × 10 ⁴	25	ns ^d	−	−	−	Meningoencephalitis; nephropathy; ocular hemorrhage; and F1 ⁺
3	7.0	5.8 × 10 ⁴	25	−	−	−	−	Pulmonary edema and hemorrhage; colitis; peritonitis; cerebral inflammation; and F1 ⁺
4	6.0	2.0 × 10 ⁵	6	+	+	+	+	Pulmonary edema and hemorrhage; hepatic fibrin; enteric and meningeal congestion
5	5.3	2.9 × 10 ⁵	10	+	nd ^e	nd	nd	Pulmonary edema and fibrin; fibrin thrombi; dermal hemorrhage
6	6.0	1.3 × 10 ⁶	9	+	+	+	+	Pulmonary edema, fibrin and perivascular hemorrhage; fibrin deposition
7	5.5	8.1 × 10 ⁷	16	−	+	+	nd	Bronchopneumonia; splenitis; typhlocolitis; meningoencephalitis
8	5.7	8.3 × 10 ⁷	6	ns	ns	ns	ns	Pulmonary perivascular hemorrhage; fibrin deposition; no specimens available; unknown cause of death

^a Two of the three non-survivors with negative cultures (in bold) had pathologic and immunohistologic evidence for disease associated the *Y. pestis* infection. Specimens suitable for analysis were not obtainable from monkey 8 due to the duration between death and necropsy. Refer to the pathology summary in Table 5.

^b Specimen positive (+) or negative (−) for growth of *Y. pestis* from cultures of indicated specimen.

^c TTD—time to death (day) post-exposure to C092 Pgm[−].

^d ns:Specimen not available.

^e nd:Not done.

Pgm[−] strain-infected African green monkeys, as characterized by the presence of a serous and fibrinous transudate, occasionally with hemorrhage, in the airways and alveolar spaces of the lethally infected animals (Fig. 1b). Although F1 antigen-positive bacteria were detected in the splenic red pulp in the majority of African green monkeys infected with the Pgm[−] strain (75%), only 1/8 (12.5%) of these animals also exhibited splenic inflammation and necrosis (Fig. 3 and Table 5). Finally, >1/3 (38%) of monkeys infected with the Pgm[−] strain had gross and microscopic lesions in the central nervous system (CNS, Fig. 4). CNS disease in the Pgm[−]-infected animals was characterized by meningitis, meningoencephalitis, and inflammation of the ventricles of the brain. The average time to death in seven animals exposed to the Pgm[−] C092 for which specimens were available was 14.6 days (Table 3).

Table 4
Summary of animals surviving aerosol exposure to C092 Pgm[−]

Number of survivors per total number exposed	Weight range, (kg)	Monkey	Inhaled dose (number of cfu)
8/16 ^a	5.5–7.0	9	3.2 × 10 ⁴
		10	6.1 × 10 ⁴
		11	4.7 × 10 ⁵ ^b
		12	1.0 × 10 ⁶
		13	1.7 × 10 ⁶
		14	1.8 × 10 ⁶
		15	5.6 × 10 ⁷
		16	8.3 × 10 ⁷

^a All 16 animals were males, both the eight that died and the eight that survived after exposure to C092 Pgm[−].

^b A peripheral blood culture collected from this monkey on day 8 post-exposure was sterile.

3.3. The virulence and immunogenicity of the C092 Pgm[−] Pla-strain in monkeys and mice

We isolated a derivative of the C092 Pgm[−] strain deficient in the production of a second major virulence factor, plasminogen activator. The two mutations in the C092 Pgm[−] Pla[−] strain were the only known alterations present. The presence and size of the three plasmids characteristic of *Y. pestis*, pFra, pLcr, and pPst were verified by plasmid extraction and gel electrophoresis, and expression of biologically active V antigen was confirmed by using a macrophage apoptosis assay. The latter detected apoptosis, induced in macrophages exposed to *Y. pestis*, that could be inhibited by pre-incubating V-producing *Y. pestis* with anti-V Ab (Weeks, manuscript submitted).

In mice, the C092 Pgm[−] Pla[−] strain was significantly more attenuated than was the parental Pgm[−] strain, all mice exposed s.c. to the highest dose tested of the double mutant (10⁸ cfu) survived (Table 2). To determine the effect of the additional mutation on the virulence of C092 Pgm[−] for monkeys, four animals were exposed by the aerosol route to high doses of C092 Pgm[−] Pla[−]. All four monkeys exposed to inhaled doses ranging from 3.5 × 10⁷ to 1.8 × 10⁸ cfu survived (data not shown), and they showed only mild symptoms of infection. During the 1st week post-exposure, the animals appeared to be depressed for approximately 2 days and exhibited a similarly transient decrease in appetite. After the first week, they resumed levels of activity and consumption which were indistinguishable from uninfected animals. In addition, the monkeys responded immunologically to infection with the double mutant. All four developed increased titers of anti-F1 Ab, with geometric mean antibody titers ranging from 5120 to 20,480, in sera collected

Table 5

Major pathologic findings in monkeys lethally-exposed to C092 Pgm[−] in comparison to those observed previously in monkeys exposed to the wt strain

	C092 Pgm [−] (n = 8) (%)	C092 wt ^a (n = 15) (%)
Gross findings		
Multilobar pneumonia	12	100
Meningitis	38	0
Microscopic findings		
Bacteriologic and antigenic		
Bacteriology		
Culture-positive blood and/or organs	71 ^b	100
Histopathology		
Disseminated bacteria in blood and/or lymphatics	88	100
Immunohistology		
F1 antigen	88	100
Pathologic		
CNS lesions ^c	38	7
Lung		
Bronchopneumonia	12	100 ^d
Pulmonary vascular leakage	50 ^e	0
Spleen red pulp		
F1 ⁺ bacteria alone	75	93
F1 ⁺ bacteria with necrotizing splenitis	12	93

^a Data reported by Davis et al. [8].

^b Based on seven monkeys for which specimens could be obtained, none was available for culture from one monkey.

^c CNS disease included meningitis, meningoencephalitis, and inflammation of the ventricles of the brain.

^d Bronchopneumonia in wt strains was characterized by neutrophilic inflammation, necrosis, hemorrhage, and massive numbers of F1 positive bacilli.

^e Pulmonary involvement was characterized not by bronchopneumonia, but by vascular leakage into alveoli and airways.

6–8 weeks after exposure. A fifth animal was included in this study to verify the susceptibility of these monkeys to the wt parental strain and the pathology associated with infection. It received an inhaled dose of 2×10^5 cfu (787 LD₅₀ doses) and died 4 days post-exposure.

4. Discussion

Live attenuated *Y. pestis* strains have long been used as plague vaccines. Most of these strains exhibit the Pgm[−] phenotype. Pigmentation in *Y. pestis* is defined as the ability to adsorb hemin or the structurally similar dye Congo red. Loss of pigmentation occurs at a high frequency in the laboratory (10^{-5}) and is now known to be due to usually the spontaneous deletion of a 102 kb fragment of the chromosome [10]. Pgm[−] strains are frequently attenuated in mammals injected s.c., and their reduced virulence is attributed primarily to the loss of an iron storage and uptake system encoded within the Pgm locus. This system is encoded by several genes within a region of the *pgm* locus termed the high-pathogenicity island (HPI) [28]. The loss of the HPI renders the organisms unable to acquire iron efficiently from the mammalian host. Because of this attenuation, Pgm[−] strains are considered to be avirulent in humans and have been used as live vaccines (as typified by the EV76 strain and its derivatives) [29]. However, their safety may be host-specific and is still the subject of debate [11–13].

Attenuated live strains of *Y. pestis* have been used as live plague vaccines in several animal species as well as in humans, however, they often caused adverse reactions. In mice, nonpigmented strains are generally virulent by the i.v. route but attenuated s.c. The Pgm[−] derivative of *Y. pestis* strain C092 had a s.c. LD₅₀ for Swiss Webster mice of 10^7 cfu (Table 2). A dose of 10^6 cfu C092 Pgm[−] given s.c. was generally nonlethal but still permitted limited replication, as detected by spleen culture. This dose also protected mice against s.c. challenge with the fully virulent strain. However, vaccine-related deaths occurred occasionally, and induration was sometimes observed at the site of inoculation in surviving vaccinees. Similarly, the EV strain showed some virulence by peripheral routes (s.c.) for some species of non-human primates such as the African green monkey (*C. aethiops*), but not for rhesus monkeys or guinea pigs [11,13,30]. Live Pgm[−] EV vaccine strains have been used worldwide as plague vaccines, however, severe local and

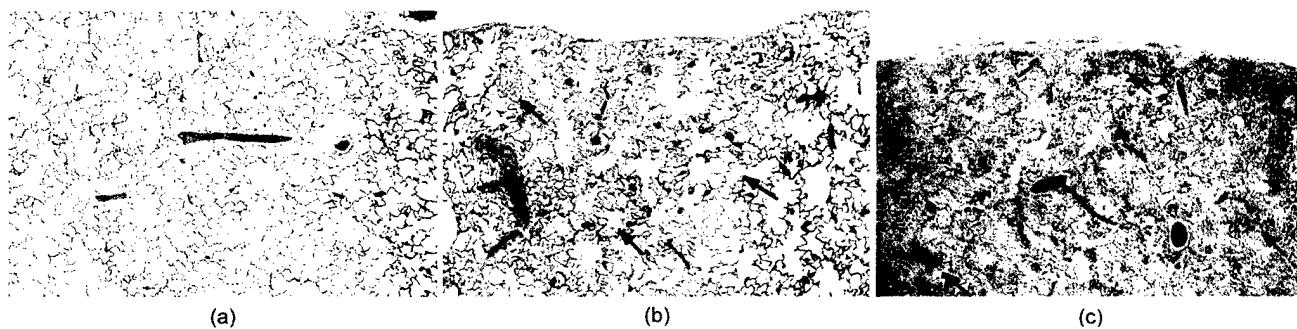


Fig. 1. Typical lung histology of normal uninfected and *Y. pestis*-infected monkeys: (A) normal monkey, hematoxylin-eosin (HE) (40 \times); (B) monkey exposed to *Y. pestis* C092 Pgm[−] with vascular leakage, the common pathology associated with lethal infection with C092 Pgm[−] shows the widespread serous and fibrinous exudate (arrows) in lung, and increased numbers of alveolar macrophages (not evident at this low magnification) HE (40 \times); (C) pneumonic plague in lung of monkey exposed to wt *Y. pestis* C092 shows typical diffuse necrotizing pneumonia in monkey infected with C092 wt. Alveoli and airways are filled with bacilli, inflammation, and hemorrhage (arrows). HE (40 \times).

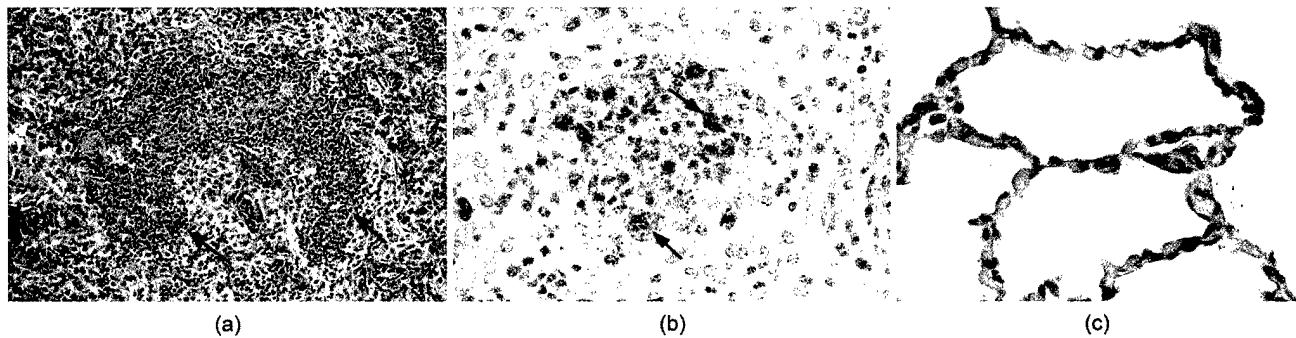


Fig. 2. Lung of C092 Pgm[−]-infected monkey with pathology resembling pneumonic plague: (A) bronchopneumonia like that found in wt-infected animals was observed in monkey #7 infected with C092 Pgm[−], neutrophils filled airway and alveoli (arrows), HE (200 \times); (B) same as (A), immunostained lung. A focus of alveolar inflammation with extracellular bacteria and bacteria within macrophages (arrows), anti-F1 immunostained (600 \times); (C) lung of normal uninfected monkey, immunostained (600 \times).

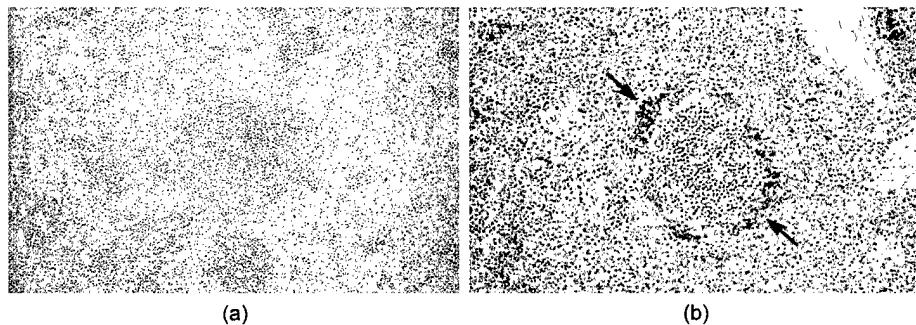


Fig. 3. Spleen of normal monkey and one exposed to C092 Pgm[−]: (A) normal uninfected monkey spleen negative for immunostained (100 \times); (B) C092 Pgm[−]-infected monkey positive for F1-immunostaining around lymphoid tissue (arrows) (200 \times).

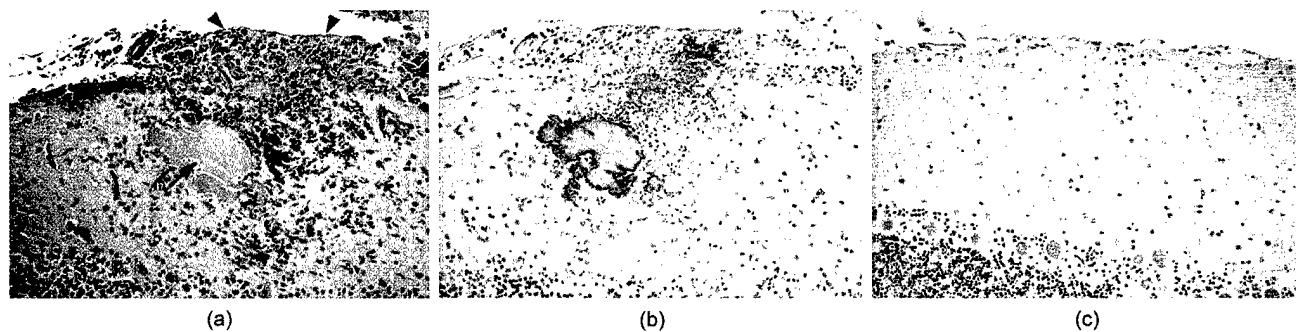


Fig. 4. CNS of monkey exposed to C092 Pgm[−], and a normal monkey: (A) brain of animal aerosol-exposed to C092 Pgm[−] with cerebellar meningoencephalitis. Acute inflammation (arrowheads), necrosis, and hemorrhage were observed, as were microcolonies (arrow), HE (200 \times); (B) the immunostained locus described in (A) stained strongly F1-positive (200 \times); (C) normal uninfected cerebellum, immunostained (200 \times).

systemic reactions associated with such strains in people have been documented with high frequency [12].

Despite their drawbacks, there is ample evidence that live attenuated strains of *Y. pestis* should be considered as potential vaccine candidates. The killed whole-cell plague vaccine previously licensed for human use protected laboratory animals against a parenteral plague, but provided little if any protection against an aerosol challenge [1–5,30–32],

epidemiologic data suggested the same possibility for humans [30,31,33]. Live vaccines (primarily Pgm[−] strains) were often more immunogenic and protective than killed vaccines in rodents [13,30,34,35]. In addition, Chen et al. [35] showed that oral administration of a live vaccine could provide a safe and effective route of vaccination for monkeys. African green monkeys given only one dose of EV76 were protected against virulent challenge, exhibited

no adverse effects, and developed serum anti-F1 antibodies. Antibodies to F1 appeared to correlate with immunity, even to the pneumonic form of plague. Byvalov et al. [18] studied the efficacy of a live Pgm^- vaccine in baboons and found that maximal protection and anti-F1 capsule titers were achieved by an initial aerosol dose of live vaccine followed by a s.c. boost of live vaccine.

Although the relative virulence of Pgm^- strains given i.p., s.c., and i.v. has been assessed in various animal models, the aerosol infectivity of such strains has not been well documented. As described above, the Pgm^- EV76 live vaccine strain was administered safely by inhalation to baboons, but the strain used was not characterized genetically [18]. Also, Soviet workers reported that vaccination of humans by the inhalation route with the NIIS derivative of the Pgm^- EV strain, was the simplest, safest, and most effective route tested [18]. In contrast, cultures available to collaborative research centers of strains of *Y. pestis* reported to be isolated from patients during the recent outbreak in India of pneumonic plague appeared to be Pgm^- ([15–17]; Chu, et al., unpublished), although the Pgm phenotypes of the original isolates are unknown, the possibility that they were Pgm^- leaves open the question of the aerosol virulence of Pgm^- strains for humans. Similar questions were raised with the finding that, in mice, the defined mutant strain C092 Pgm^- exhibited a LD_{50} aerosol dose was about a magnitude less (10^6 cfu) than the s.c. LD_{50} (Table 2). It is possible that sensitivity to Pgm^- strains of *Y. pestis*, depends on the species and strain of host as well as on the strain of attenuated *Y. pestis* and the route of administration [11,13,35].

Many of the problems associated in the past with the use of live attenuated plague vaccines, as alluded to above, were probably due to their undefined nature. For instance, difficulties in maintaining stocks of Pgm^- strains of standardized efficacy and virulence have been reported, strains have reportedly lost their immunogenic effects, possibly because of mutation or plasmid loss, and other strains were able to revert to virulence by animal passage [11–13,30]. Many variants of the classical EV-type strain exist in different laboratories. One investigation of phenotypic differences exhibited by different derivatives of EV76 found that all were Pgm^- , but they varied in their synthesis of the F1 antigen, calcium-dependent growth, activities associated with the pPst-encoded plasminogen activator, and other virulence-associated characteristics [30]. Development of a live strain possessing defined, non-reverting and attenuating mutations in known virulence-associated loci would obviate many of the difficulties accompanying use of the previous strains and render them safer to administer as vaccines and to handle in non-containment laboratories.

The purpose of this study was to determine the virulence by the aerosol route of both a defined Pgm^- mutant of the C092 strain of *Y. pestis* and of a Pla^- derivative of the latter in the non-human primate (*C. aethiops*) model of human pneumonic plague.

The Pgm^- mutant of *Y. pestis* strain C092 was clearly virulent in the African green monkey, killing half of the 16 animals challenged by the aerosol route. These data agree with that of previous studies that demonstrated the virulence by peripheral routes of EV76-type strains of attenuated *Y. pestis* for some species of non-human primates, including African green monkeys (*C. aethiops*), langurs, and grivets, but not rhesus monkeys (*Macaca mulatta*) and some other species of *Macaca* [11,13], the latter have significant individual variability in their susceptibility to fully virulent *Y. pestis* when exposed by various routes [13]. Hallett et al. [11] found that, although a strain designated EV51f was nonlethal by the s.c. route at doses up to 10^8 bacilli in guinea pigs, it was pathogenic for *C. aethiops*, causing death in 26% of monkeys inoculated s.c. Pathological changes were generally similar to those observed in a virulent *Y. pestis* infection, as measured by bacteremia, temperature and leukocytes increases, and postmortem findings typical of bubonic-septicemic plague. Interestingly, as shown in this report, there was no dosage–lethality relationship, as even doses as low as 100 bacilli could invade, multiply, and kill [11].

In this study, three of five animals died at the lowest inhaled doses ranging from 0.9×10^4 to 6.1×10^4 cfu. This compares with an aerosol LD_{50} of 3.4×10^2 cfu for the wt C092 strain (Pitt and coworkers, unpublished data). Thus, while the Pgm^- strain is significantly less virulent than the wt strain, it retains the ability to cause lethal infection by the aerosol route and is not avirulent. The deaths of five of the animals lethally exposed to the C092 Pgm^- strain were clearly attributed to the *Y. pestis* infection, as determined by culture, and all except one of the non-survivors had pathologic and immunologic evidence of infection with the Pgm^- strain (Tables 3 and 5). There were both differences and similarities in the disease associated with the lethal infection of the Pgm^- compared to the wt strain of *Y. pestis*. As observed in infection with the wt strain, the Pgm^- organisms were apparently disseminated widely, as shown bacteriologically and by immunohistologic detection of the F1 capsule antigen in the blood and various organs. Nevertheless, there were significant differences in the pathology of the lethal infection. Most notably, the prominent pneumonia observed after aerosol exposure to wt *Y. pestis* was only seen in one of the animals lethally exposed to the Pgm^- strain, and the higher incidence of CNS disease (meningitis and inflammation of the brain) documented in the Pgm^- -infected non-survivors was not a notable finding in *C. aethiops* exposed by aerosol to the wt strain (Table 5; [8]). This may relate to the more prolonged time to death (mean of 14.6 days, Table 3) in the Pgm^- compared to the wt C092 (mean of 5.6 days in animals exposed to doses of 10^2 – 10^5 cfu) [8]. It is also of interest that the two animals with the longest time to death (25 days), monkey 2 and 3 (Table 3), had evidence of meningitis. Blood, lung, liver, and spleen samples from these animals were negative by culture and anti-F1 antibody (Tables 3 and 5). However, although the CNS was

not cultured, the CNS lesions of the monkeys were positive for staining with the anti-F1 antibody. This suggests that the animals might have cleared the infection if meningitis had not occurred.

In addition to the C092 Pgm[−] strain, another live vaccine candidate is an attenuated derivative of C092 that is unable to produce Pla. Pla is encoded on the pPst plasmid found in virulent strains of *Y. pestis*. It is a major virulence factor and has been proposed to facilitate the spread of the organisms from peripheral sites of infection [21]. Strains cured of the pPst plasmid retained their virulence if inoculated by a direct route such as i.v., however, loss of pPst led to a million-fold increase in s.c. LD₅₀ in mice [21,22]. Consequently, pPst-cured or other Pla[−] mutants are reasonable live vaccine candidates. We isolated and characterized a pPst-cured derivative of C092. It replicated at the s.c. inoculation site, but showed only limited, low-level replication at distant sites such as lymph node and spleen [22]. Thus, it might stimulate a vigorous immune response in the host. In addition, the Pla protease does not appear to be an effective immunogen in mice, suggesting that its absence in pPst[−] strains would not greatly decrease the protective efficacy of such strains. The aerosol infectivity of pPst[−] strains has not been extensively characterized, in mice, the aerosol LD₅₀ of pPst-cured C092 was four-fold greater than that of the wt strain (Table 2).

Introduction of the mutation in the *pla* locus appeared to increase the attenuation of the Pgm[−] strain. None of the four monkeys exposed to high aerosol doses succumbed to the infection. Although post-exposure blood samples were not cultured, serological data indicated that the organism appeared to have multiplied at least transiently. The serological responses of the monkeys exposed to the *Y. pestis* C092 Pgm[−] Pla[−] strain demonstrated that, despite its significant attenuation, a single dose of the double mutant was able to induce an immune response in all four exposed animals who developed antibody titers ranging from 1:2560 to 1:20,480 to the F1 antigen.

In summary, in this study, the C092 Pgm[−] strain caused lethal disease in 50% of non-human primates exposed by aerosol. In contrast, all animals exposed to high doses of the C092 Pgm[−] Pla[−] mutant survived, and the double mutant induced an immune response. Thus, the results suggest that Pgm[−] strains retain some virulence by the aerosol route for *C. aethiops*, and that defined multiply attenuated strains of *Y. pestis* may be potentially safe and efficacious plague vaccine candidates.

Acknowledgements

The authors thank Larry Ostby for assistance in figure preparation, Steven Töbery for photomicroscopy, Chris Bolt for ELISA titer determinations, and Kathryn Kenyon, Joel Bozue, and William Day for their helpful reviews.

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